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Genetics

Hypermutation at the immunoglobulin heavy chain locus in a pre-B-cell line

(amber termination codon/mutation/mutator/compartmentalization test)

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ABSTRACT **Most cells in the well-known pre-B-lymphocyte line 18-81 have correctly assembled genes for both alleles at the immunoglobulin heavy chain locus. Only one allele is "active"; the other, "silent" allele contains an amber termination codon. The rate of reversion of this amber codon was determined to be 0.3–1 × 10^{-4} per cell generation. This high rate is termed hypermutation.**

Higher vertebrates can synthesize a vast number of antibody molecules that are formed by combining immunoglobulin heavy (H) and light (L) chains, each of which bears one out of a vast number of variable (V) regions. In the germ line, there are no complete genes for the Ig chains, only gene segments. At the H chain locus in the mouse, there are *ca.* 100 V_H, *ca.* 20 diversity (D_H), and 4 joining (J_H) gene segments. In pre-B-cells, an H chain gene has been formed by joining together one gene segment of each type (reviewed in ref. 1). Thus, there is a large combinatorial diversity, and further diversity is generated by variations in the precise points of juncture. But this is not enough, and it is generally accepted that somatic mutation, probably point mutation, must also contribute to the diversity of antibody molecules (reviewed in ref. 1). However, it is not known whether somatic mutations are selected from among those that arise at the "normal," spontaneous rate or whether the mutation rate, for the immunoglobulin loci at least, is increased at some period during the ontogeny of B lymphocytes. If there is hypermutation, it is not clear whether it is driven by antigen—hence limited to B cells—or whether it is independent of antigen—hence possibly acting before the B-cell stage. Here we report measurements of a mutation rate that lead us to conclude that there is hypermutation in the pre-B-cell line 18-81.

Cells of the pre-B-cell line 18-81 synthesize only H chains (2, 3), which can be detected only in the cytoplasm. The L chain loci are usually in the embryonic configuration; that is, their gene segments have not been joined to form functional L chain genes (4). The line is diploid for chromosome 12 (3), which carries the H chain locus (5, 6). In both homologous chromosomes, the *V*, *D*, and *J* gene segments have been correctly joined to form H chain genes (7). Since the H chain alleles contain either J_{H2} or J_{H3} (7, 8), we call the alleles *V2* and *V3*, respectively (9). The cells of the 18-81 line usually produce H chains encoded by the *V3* allele only (4, 7, 8), because the *V2* allele contains an amber nonsense codon in the *D* segment (10) that prematurely terminates translation. During culture, some cells switch from synthesizing μ heavy chain to γ2b heavy chain (4). In the γ2b-producing cells, DNA sequences, including the C_μ gene segment that was previously expressed, have been deleted (4). A few of the cells that produce γ2b chain also produce μ chain (3), which, in this case, is encoded by the usually silent allele (8).

MATERIALS AND METHODS

Compartmentalization Test. Single cells were distributed into microtiter plates at limiting dilution (0.15 cell per well) without feeder cells. The medium was supplemented with hybridoma growth factor (11) to support the growth of single cells. When the clone had reached the size of 2–3 × 10^3 cells, the cells were harvested and assayed for cytoplasmic μ chain by immunofluorescence (3). The immunofluorescent intensity of the positive cells was high and very uniform, both within and between clones.

DNA Sequencing. DNA from the hybridoma H3.3.1.1.83.13 was isolated and digested with the enzyme *Eco*RI. A 2.4-kilobase (kb) fragment was cloned into the phage λ1150. A 0.4-kb *Bam*HI–*Eco*RI fragment containing most of the V_H, D_H, and J_{H2} segments was subcloned into phage M13 vectors *mp*8 and *mp*9 (12), and both DNA strands were sequenced as described by Sanger *et al.* (13).

Cell Fusion. Clone A3.2 was fused with the myeloma Ag8653 as described (3).

RESULTS AND DISCUSSION

Reversion of a Termination Codon Allows Expression of the Usually Silent Allele. There are several ways in which the arrest of translation due to the amber codon might be overcome: (*i*) recombination (either reciprocal or "conversion"), (*ii*) reading through the stop codon during translation ("suppression"), or (*iii*) mutation either by an in-frame deletion or by reversion to a codon specifying an amino acid. To decide among these possibilities, we determined the nucleotide sequence of the *V2* allele in the subclone H3.3.1.1.83.13 (Fig. 1), which produces both γ2b and μ chains (8). We found that the termination codon TAG present in the *D* gene segment of the *V2* allele in the 18-81 pre-B-cell line had changed to the codon TAC (specifying tyrosine) in the double-producing subclone (Fig. 2). We also found another mutation in the V_H gene segment: at triplet position 42, the codon GAG in the germ-line V_H gene segment (14) that was used in creating the *V2* allele had changed to GAA in the cell producing both γ2b and μ chains. The *V2* allele of the 18-81 line could not have been exchanged, because restriction fragments containing the *V2* allele are the same in the parental line and in the variant, double-producing subclone. Furthermore, the codon TTG at triplet position 47 is diagnostic for this particular V_H gene segment (14).

While the preponderance of evidence leads us to believe that the reversion is the result of point mutation, we cannot

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Abbreviations: H, heavy; L, light; V, variable; D, diversity; J, joining; C, constant.

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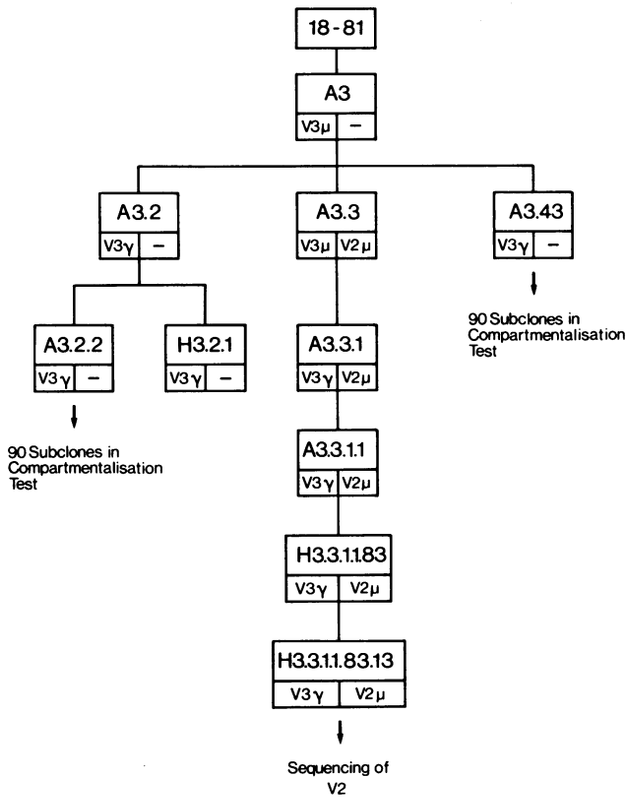

FIG. 1. Genealogy of subclones and hybridomas derived from the Abelson virus-transformed line 18-81. Abelson virus-transformed cells are designated "A" and their hybridomas, "H." The heavy chains produced by the lines are given below the subclone designation. V2 and V3 are the variable regions of the heavy chains.

completely rule out a mechanism involving close double exchange (either reciprocal or "conversion"). Comparing our sequence of the intron downstream from (i.e., 3′ to) the J_{H2} segment to the sequence determined by Newell *et al.* (29),

30	CCT	TCC	CAT	GAC	ATG	TCT	TGG	GTC	CGC	AAG
40	ACT	CCG	GAA[a]	AAG	AGG	CTG	GAG	TTG	GTC	GCA
50	GCC	ATT	AAT	AGT	GAT	GGT	GGT	AGC	ACC	TAC
60	TAT	CCA	GAC	ACC	ATG	GAG	AGA	CGA	TTC	ATC
70	ATC	TCC	AGA	GAC	AAT	ACC	AAG	AAG	ACC	CTG
80	TAC	CTG	CAA	ATG	AGC	AGT	CTG	AGG	TCT	GAG
90	GAC	ACA	GCC	TTG	TAT	TAC	TGT	GCA	AGA	CAT
100	GAC	TAC[b]	TAT	GGT	AAC	TTC	TGG	GGC	CAA	GGC
110	ACC	ACT	CTC	ACA	GTC	TCC	TCA	GGT	GAG	TCC
120	TTA	CAA	CCT	CTC	TCT	TCT	ATT	CAG	CTT	AAA
130	TAG	ATT	TTA	CTG	CAT	TTG	TTG	GGG	GGG	AAA
140	TGT	GTG	TAT	CTG	AAT	TTC	AGG	TCA	TGA	AGG
150	ACT	AGG	GAC	ACC	TTG	GGA	GTC	AGA	AAG	GGT
160	CAT	TGG	GAG	CCC	TGG[c]	CTG	ATG	CAG	ACA	GAC
170	ATC	CTC	AGC	TCC	CAG	ACT	TCA	TGG	CCA	GAG
180	ATT	TAT								

FIG. 2. Partial sequence of the *V2* allele of hybridoma H3.3.1.1.83.13. Triplets are numbered as described by Yancopoulos *et al.* (14). Triplets of interest are underlined: a, germ-line and 18-81 triplet is GAG (14); b, 18-81 triplet is TAG (10); c, BALB/c triplet is TAG (29).

there is yet a third change at triplet position 164 from TAG to TGG (Fig. 2). We do not know whether this difference is the result of a mutation in the history of the 18-81 line or of substrain differences in the BALB/c mouse strain from which the 18-81 cell line arose.

Rate of Reversion. Although we did not determine at which point in the history of the 18-81 subclones the codon GAG changed to GAA, the reversion of the amber termination codon is easily localized—it must coincide with the expression of μ chain from the usually silent *V2* allele. In cell lines that produce either no H chain or a γ2b chain encoded by the *V3* allele, rare variants that synthesize μ chain encoded by the *V2* allele can be determined by immunofluorescence microscopy. Most of the clones that have switched to γ2b chain production also contain μ chain-producing cells at a frequency of about 0.5% when the clone reaches a size of $\approx 5 \times 10^7$ cells (8). This frequency was usually maintained after repeated subcloning of the γ2b chain-producing clones.

Using a compartmentalization test (15), we have determined the mutation rate for two independent subclones, A3.2.2 and A3.43 (Fig. 1). Two subclones that arose from preexisting mutants in the population, one from A3.2.2 and one from A3.43, were grown to higher cell numbers, and their μ chains were analyzed by $NaDodSO_4$ gel electrophoresis. The μ chain produced by these subclones was identical in position and amount (as determined visually) with that produced by A3.3.1.1.

The clones that did not arise from preexisting mutants were subjected to the following analysis. The average number of mutational events per well, m, is given by

$$e^{-m} = N_0/N,$$

where N is the number of wells in an experiment and N_0 is the number of wells with no revertants.

The number of mutational events per cell per generation, M, is then given by

$$M = m/2C,$$

where C is the number of cells per well (15). When the number of cells varies considerably from well to well, it is possible that the use of an average value for C might lead to an erroneous estimate. To avoid this, we define

$$m_i = 2\,MC_i,$$
$$q_i = e^{-m_i},$$

and

$$p_i = 1 - e^{-m_i},$$

where m_i is the expected number of mutational events in a well with C_i cells, q_i is the probability that this well has no mutant clones, and p_i is the probability that there are one or more mutant clones.

The likelihood of obtaining the observed experimental results is then

$$L = \prod_{i=1}^{i=N-N_0} p_i \cdot \prod_{j=1}^{j=N_0} q_i.$$

Taking logarithms and differentiating, we get

$$Q = \frac{\partial \ln L}{\partial M} = \sum_{i=1}^{i=N-N_0} 2C_i q_i/p_i - \sum_{j=1}^{j=N_0} 2C_j,$$

where the subscript i refers to single wells with one or more

Table 1. Mutation rate measurements

	Subclone	
	A3.43	A3.2.2
Total wells	48	88
Positive wells, no.	27	10
Negative wells, no.	21	78
Cells per well, $\overline{no.}$	3396	1938
Cells per positive well, $\overline{no.}$	3557	2635
Cells per negative well, $\overline{no.}$	3189	1849
Maximum likelihood estimate of mutation rate $\hat{M}$ per cell generation	1.3×10^{-4}	3.2×10^{-5}

mutant clones and the subscript j refers to wells with no revertants. The maximum likelihood estimate of the mutation rate $\hat{M}$ is obtained by setting Q to zero. Although no closed form solution is possible, the equation $Q = 0$ is easily solved on a digital computer by using a binary search algorithm.

Since most mutational events at each of the three base pairs in the amber codon should lead to a detectable reversion, we divide the rate $\hat{M}$ from Table 1 by 3 to get the mutation rate per base pair per cell generation; this rate is 4.2×10^{-5} for clone A3.43 and 1.1×10^{-5} for clone A3.2.2.

Clone-Size Distribution and the Delay of Gene Expression. In Fig. 3 we present a Luria plot of the distribution of mutant clone sizes. The distribution curve deviates somewhat from the theoretically expected line (16); there are relatively too few clones of size 1. Obviously, small clones would be more subject to losses during the experimental procedure, but we do not think that such losses are an important source of error. Another reason for the deviation is the finite multiplicity; that is, many of the wells must contain revertants that arose from more than one mutational event. The deficit in small clones probably also results from phenotypic lag—i.e., a delay in gene expression. If there is a delay of a generation or so between the time when the mutation occurs and the time when sufficient H chain has accumulated to be detectable by immunofluorescence, the mutation rate per base pair per cell generation could be up to twice as high as our estimate.

The Case for Hypermutation. The mutation rate in the *D* gene segment of the *V2* allele in the pre-B-cell line 18-81 should be compared to some "normal" or control mutation rate. Unfortunately, as far as we are aware, there are no direct measurements of mutation rates per base pair per generation in mammals, much less in cell lines of BALB/c mice.

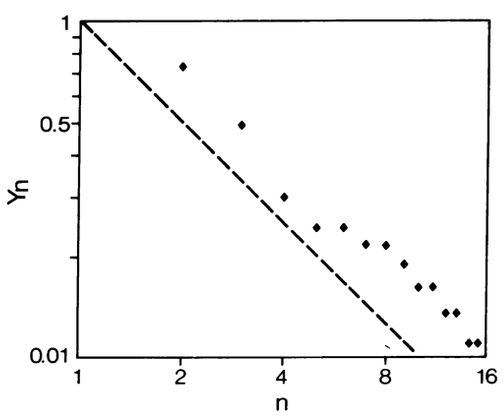

FIG. 3. Luria plot of the distribution of mutant clone sizes. The abscissa is the clone size, n; the ordinate is the frequency with which clones of size n or greater are found. Both scales are logarithmic. The points are data pooled from both experiments described in Table 1. The broken line is the theoretical distribution of Luria (16).

Nevertheless, it is clear that *V2* reverts at an extraordinarily high rate.

In yeast, mutation rates are of the order of 10^{-8} per codon per generation (15). The yeast genome is several orders of magnitude smaller than that of the mouse. But the larger the genome, the smaller the mutation rate must be; otherwise, a stable wild-type sequence could not be maintained. Natural selection cannot work when all offspring are multiply mutant. Therefore, we argue that the rate in the mouse can be no higher than 10^{-9} per base pair per generation. On the basis of the population genetics of hemoglobin variants in man, it has been concluded that the spontaneous mutation rate is 10^{-9} to 10^{-8} per base pair per organism generation (17); thus, the rate per base pair per cell generation must be $<10^{-9}$. Rates of spontaneous mutation to resistance to the lethal effect of ouabain have been measured in mouse cell lines: 5×10^{-8} per cell generation in a pre-B-cell line and 6×10^{-9} in a thymoma line by R. C. von Borstel (18). These rates were determined for a whole gene—that is, mutations at an undetermined number of nucleotide sites give rise to the observed phenotype. Thus, these data are consistent with 10^{-9} as an upper limit for the rate per base pair. This rate is lower by a factor of 10,000 than the rate of $\approx 10^{-5}$ that we measured for the *V2* allele of the 18-81 cell line.

The proximate cause for the hypermutation in this cell line we call the "immunoglobulin mutator system." The mutator system could be a special enzyme complex that actively causes mutations (19). On the other hand, it could act by deactivating the error–correction and error-free repair systems that normally function in eukaryotic cells, in which case the high error rate of DNA polymerase would be sufficient to cause hypermutation. But if hypermutation results solely from generalized suppression of error–correction and of repair, the mutator system must act on the entire genome. Experiments are underway to define the scope of action of the mutator system.

The Immunoglobulin Mutator System Also Acts in Cells That Have Not Switched in H Chain Class Production. A few years ago, it was suggested that somatic mutation only occurs in cells that express an H chain different from μ chain (20, 21). Since then, variant μ chain V regions that result from somatic mutation have been reported (22). The mutation rates given above were determined in cell clones producing γ2b chain derived from the usually active allele. However, we also did compartmentalization tests with subclones A3.8 (4) and A3.13, both of which have lost the *V3* gene segment, but still contain both C_μ alleles. The mutation rates for these two clones were both $>10^{-5}$ per base pair per generation (data not shown). Thus, hypermutation can occur in cells that have not switched in H chain class production. Nevertheless it could be argued that the action of the mutator is coupled to the enzymatic activity catalyzing the H chain class switch and that these enzymes are present in all the cells of the 18-81 cell line.

The Immunoglobulin Mutator System Is Not Active in a Myeloma and Its Hybridomas. Several workers have failed to find V region variants at high frequency in myeloma and hybridoma proteins (23–26), although there exists one case where the mutator system may still be active (27). To determine whether the mutator system is active at the plasma cell stage, we fused subclone A3.2, a γ2b chain producer (Fig. 1), with myeloma Ag8653, which does not produce any Ig chain. Of 20 hybridomas, 18 produced γ2b chain alone, and 2 produced no H chain (4). The rate of H chain synthesis, the steady-state level of H chain in the cytoplasm, and the steady-state level of mRNA specific for this H chain are the same as in plasma cell hybridomas (9, 28). Some of the hybridomas also produce κ chain and secrete complete Ig molecules. We screened a total of about 2×10^6 cells (from clones that had been expanded to at least 5×10^8 cells) by immuno-

fluorescence and did not detect any cells producing μ chain. In one of the hybridomas, H32.1 (Fig. 1), we established that mRNA specific for μ chain was transcribed (unpublished data), so an active μ chain gene must still have been present. Therefore, we conclude that the mutator system is not active in a myeloma and its hybridomas.

The 18-81 cell line and its subclones are quite unstable genetically, but their hybridomas are as stable as any other cell lines. Indeed, we were able to sort out the stages of differentiation that cells of these lines go through only by generating hybridomas at each stage, thereby fixing the genetic and physiological state (3, 4). In addition to deletions involved in the switch in H chain class production, 18-81 subclones accumulate deletions, particularly in the major intron (i.e., between J_H and C_μ). The frequency with which these deletions arise is so great that DNA mapping by Southern blotting may be misinterpreted (7). It is our impression that deletions occur much less frequently in the hybridomas. We speculate that these events are related to the immunoglobulin mutator system. In any event, it seems obvious that there are limits to the stability of any cell line with an active mutator system.

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